PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number: WO 99/54359
C07K 19/00, 14/65, C07H 21/04, C12N 15/62, 15/12, 15/19, 15/16, 9/20, 9/74, A61K 38/18, A61L 27/00	A1	(43) International Publication Date: 28 October 1999 (28.10.99)

(21) International Application Number: PCT/AU99/00292

(22) International Filing Date: 19 April 1999 (19.04.99)

(30) Priority Data:
PP 2984 17 April 1998 (17.04.98) AU

(71) Applicants *(for all designated States except US)*: GROPEP PTY. LTD. [AU/AU]; Gate 11, Victoria Drive, Adelaide, S.A. 5000 (AU). BELFORD, David, Andrew [AU/AU]; 19 Thomas Street, Seacliff Park, S.A. 5049 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HUMPHRYS, Simon, Troy [AU/AU]; Mulberry Farm, Barker Road, Mount Barker, S.A. 5251 (AU). BALLARD, Francis, John [AU/AU]; 52A Bridge Street, Kensington, S.A. 5068 (AU).

(74) Agent: GRIFFITH HACK; 3rd floor, 509 St. Kilda Road, Melbourne, VIC 3004 (AU).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MATRIX BINDING FACTOR

(57) Abstract

The present invention relates to the design, manufacture and use of novel polypeptide bioactive factors. More particularly, the invention relates to the targeting of polypeptide bioactive factors to biological or chemical substrates via engineered amino acid motifs, while maintaining or extending the biological activities attributed to that polypeptide bioactive factor. The invention provides a recombinant polypeptide bioactive factor, referred to as a matrix binding factor (MBF), comprising a polypeptide bioactive factor in which the naturally–occurring amino acid sequence of the factor has been modified to introduce one or more amino acid substitutions, deletions and/or additions which increase the affinity of the polypeptide bioactive factor for a negatively–charged surface. Preferably the polypeptide bioactive factor is a growth factor which stimulates proliferation, differentiation, migration or cellular activity, and most preferably is a member of the insulin–like growth factor (IGF) family of growth factors. The invention also provides nucleic acid molecules encoding polypeptide bioactive factors of the invention, methods for production of the factors, and compositions and methods for use of the factors in treatment of a variety of pathological conditions, for use in tissue culture and for the preparation and use of surgical implants or prostheses.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

MATRIX BINDING FACTOR

The present invention relates to the design, manufacture and use of novel polypeptide bioactive factors. More particularly, the invention relates to the targeting of polypeptide bioactive factors to biological or chemical substrates via engineered amino acid motifs, while maintaining or extending the biological activities attributed to that polypeptide bioactive factor.

BACKGROUND OF THE INVENTION

Polypeptide bioactive factors, particularly growth factors, are crucial for many cellular processes. Consequently, much research is undertaken in relation to the presence and mechanism of action of such factors in cellular processes, with the objective of identifying novel polypeptide bioactive factors, identifying growth factor synergies, and understanding the mechanisms of processes such as cellular maintenance, growth, development, and apoptosis. The potential usefulness of polypeptide bioactive factors, particularly growth factors, in diagnostics, pharmaceuticals and therapeutics is well recognised.

Considerable interest has been directed to a family of polypeptide bioactive factors, the insulin-like growth factors (IGFs). It is well established in the art that these proteins are structurally related, and that their expression patterns, localisation and bioactivity are important for the differentiation and proliferation of various cellular types. This has led to the suggestion that IGFs are pivotal for cellular migration and proliferation, for connective tissue production, and for turnover processes associated with tissue remodelling, repair and wound healing (Clemmons, D.R., British Medical Bulletin, 1989 45 465-480; Gartner *et al*, J. Surg. Res., 1989 52 389-394).

Despite the demonstrated effects of IGFs in cultured vertebrate cells and tissues, as yet there have been no observations *in vivo* which support any unequivocal role in tissue remodelling or wound healing for exogenously-administered IGFs, whether given systemically, locally or topically. It is therefore accepted in the art that there must be other moieties which influence the bioavailability of IGFs.

The bioavailability of a polypeptide bioactive factor is a measure of that factor's ability to remain active at a site where it can effect a desired cellular response. Bioavailability is modulated by the stability, protease susceptibility and rate of clearance of a factor from the site where it interacts with its cellular receptors.

The bioavailability of IGFs can be modulated by one or more of the six insulin-like growth factor binding proteins (IGFBPs) which are so far known. Furthermore, the bioavailability of an IGF may also be modified by structural changes to the amino acid sequence of the polypeptide. As a hypothetical example, introducing an affinity for one or more biological or chemical substrates may serve to slow or prevent IGF clearance from the local environment, or may even localise an IGF in a biologically active and accessible form. Moreover, this general concept may be extended to polypeptide bioactive factors other than IGFs.

The nature of many biological substrates, such as basement membrane, extracellular matrix (ECM), bone matrix and other connective tissue components, is such that localised and more general patterns of negative charge are created. For example, both heparan sulphate proteoglycans present in the ECM and hydroxyapatite in bone have net negative charges. These charged moieties provide sites of attachment for factors with the appropriate affinities, as determined by their amino acid sequences. The potential of factors able to elicit cell growth effects at the site of

such localisation offers opportunities to create a significant improvement in the delivery of therapeutic agents to their preferred site of action. Similarly, therapeutic agents that are retained at the preferred site of action when administered locally may prove useful for the treatment of many conditions, such as bone or ligament grafts and tendonitis, or they may improve the post-operative recovery associated with tissue manipulations, cartilage repair and reconstructive surgery.

Gastrointestinal tract impairment associated with either iatrogenic disorders such as chemotherapy-induced epithelial damage or pathologies such as inflammatory bowel disease are significant causes of morbidity. Accordingly, therapeutic agents directed to the healing of the epithelial lining of the gastrointestinal tract are useful in the prevention and/or treatment of conditions associated with impaired gut function.

The ability of negatively-charged substrates, such as heparan sulphate proteoglycans, to protect bound polypeptides from degradation due to physical stresses such as temperature, chemical stresses such as low pH, and/or biological stresses such as protease degradation, as described in the prior art, provides potential mechanisms for the prevention and treatment of pathological states associated with impaired gut function.

Accordingly, there is a need in the medical and veterinary fields for novel delivery and targeting technologies which create local concentrations of bioactive factors.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a polypeptide bioactive factor, hereinafter referred to as a matrix binding factor (MBF), comprising a polypeptide bioactive factor in which the naturally-occurring amino acid sequence of the factor has been modified to introduce one or more amino acid substitutions, deletions and/or

additions which increase the affinity of the polypeptide bioactive factor for a negatively-charged site or surface.

The term "polypeptide bioactive factor" means a polypeptide or small protein which either modulates cellular responses directly, as is the case with growth factors, or acts indirectly by its association with other components, including the extracellular matrix, such that cellular responses are potentiated. This term is also to be understood to encompass mutants, fragments and analogues of such a factor which retain at least one of the biological activities of the factor.

The term "negatively-charged surface" means any surface which displays an array of negative charge that provides association sites for positively-charged polypeptide motifs. Such negatively-charged surfaces may be of natural or artificial origin, and may be in an animal body. They include, but are not limited to, extracellular matrix, dextran sulphate, chondroitin sulphate, dermatan sulphate, collagen, fibronectin, vitronectin, laminin, heparan sulphates, heparin, hydroxyapatite, anionic plastics, silicates, and physiologically-compatible metals and other materials used in surgical implants or prostheses, such as stainless steel, titanium, metal alloys, ceramics, polymers and plastic coated metals.

Metals and ceramics are widely used in orthopaedic applications.

As an integral part of their biological activity, a number of polypeptide bioactive factors have affinities for sites other than their high affinity cellular receptors. For example, they may bind to cell attachment factors, basement membrane moieties, extracellular matrix components, or soluble circulating proteins.

Accordingly, the amino acid modifications include introduction of sequences which are deduced from motifs in the amino acid sequences of polypeptide bioactive factors which have been identified by their particular affinity for specific substrates. Such motifs include, but are not

limited to, the heparin-binding amino acid motif characteristic of fibroblast growth factors (FGFs), heparin-binding epidermal growth factor, vitronectin, fibronectin, histidine-rich glycoprotein or purpurin.

Preferably the polypeptide bioactive factor into which changes are introduced in order to create an MBF is a polypeptide bioactive factor which does not already include similar sequence motifs which confer an ability to bind to negatively-charged surfaces.

More preferably the polypeptide bioactive factor into which changes are introduced in order to create an MBF is a growth factor which stimulates proliferation, differentiation, migration or cellular activity, including, but not limited to, members of the insulin-like growth factor (IGF), transforming growth factor-β, platelet-derived growth factor, vascular endothelial growth factor or epidermal growth factor families of growth factors.

The present invention also includes within its scope biologically-active mutants, analogues and derivatives of the MBF. Preferably such modified MBFs are in a biologically pure form.

The term "biologically pure" as used herein means a product essentially devoid of unavoidable biologically active impurities or contaminants.

According to a second aspect, the invention provides a nucleic acid molecule whose sequence encodes a polypeptide bioactive factor of the invention. The nucleic acid molecule may be a cDNA, a genomic DNA, or an RNA, and may be in the sense or anti-sense orientation. Preferably the nucleic acid molecule is a cDNA, more preferably a sense cDNA.

In a third aspect, the invention provides a method for producing a recombinant MBF, comprising the steps of subjecting a cloning vector comprising a nucleic acid sequence encoding the polypeptide growth factor to mutagenesis to generate a nucleotide sequence encoding an MBF.

The nucleic acid sequence of the polypeptide bioactive factor must be subcloned into an appropriate cloning vector or plasmid. This permits mutagenesis of the DNA encoding a polypeptide growth factor to generate a sequence encoding a MBF, by means well known to those in the art.

Preferably the mutagenesis is achieved using site-directed mutagenesis, more preferably using oligonucleotide primers which are based on binding sites able to bind to negatively-charged surfaces, which binding sites are present in other polypeptide bioactive factors, or sequences substantially homologous thereto. Examples of polypeptide bioactive factors comprising such binding sites include vitronectin, 10 kilodalton gamma-interferon inducible protein, histidine-rich glycoprotein, purpurin, beta-thromboglobulin, antithrombin III, heparin cofactor II, FGF-1, FGF-2, heparin-binding epidermal growth factor, lipocortin, protein C inhibitor, fibronectin, thrombospondin, lipoprotein lipase, hepatic triglyceride lipase, vascular endothelial cell growth factor, thrombin, neural cell adhesion molecule and glial-derived nexin.

The altered nucleic acid sequence encoding an MBF may be subcloned into a suitable expression vector, which may be introduced into host cells by conventional means familiar to those skilled in the art.

Thus the method of the invention preferably also comprises the steps of

subcloning the nucleic acid sequence encoding the MBF into a suitable expression vector;

transforming the expression vector into a suitable bacterial, yeast or tissue culture host cell;

cultivating the host cell under conditions suitable to express the MBF; and

isolating the MBF.

It will be appreciated that host cells comprising selected constructs so formed may express the MBF as a fusion protein within inclusion bodies (IB). By the term

"MBF fusion protein" we mean a polypeptide consisting of two linked protein components, one of which is selected so as to be expressed in the host cell under the control of a suitable promoter, and the other of which comprises the polypeptide bioactive factor incorporating the motif that confers MBF activity. The fusion protein is produced in order to facilitate the expression and/or processing of the amino acid sequence of the MBF activity. Preferably the MBF fusion protein is produced by an appropriate host cell in a fermenter by conventional means understood by those skilled in the art.

Preferably, the MBF is isolated from the host cell following disruption of the host cell by homogenisation, and processed to its biologically pure form using conventional methods of protein purification well recognised by those skilled in the art. These include oxidative refolding to achieve correct disulphide bonding, chemical cleavage of the fusion partner (if used) from the MBF, and various chromatographic steps. The MBF may be isolated as a biologically pure form of the fusion protein, and may then be cleaved from its fusion partner, yielding a peptide that is not extended.

In a preferred embodiment an MBF is prepared as a fusion protein using the methods described in our Australian Patent No. 633099, the entire disclosure of which is incorporated herein by reference. In this method a fragment of porcine growth hormone is linked to the N-terminal sequence of an MBF, optionally via a cleavable sequence.

In another preferred embodiment of the invention there is provided a process for the production of a cleavable MBF fusion protein, comprising the step of transforming a susceptible bacterial, yeast or tissue culture cell hosts with one or more recombinant DNA plasmids which include DNA sequences capable of facilitating the expression of an MBF fusion protein.

In a further preferred embodiment, the MBF fusion protein is expressed as an insoluble aggregate or inclusion body within the host cell, and isolated by cell disruption and centrifugation. The conventional methodologies which may be employed in the isolation of the MBF include fusion protein dissolution, oxidative refolding, hydroxylamine or proteolytic cleavage, and various chromatographic processes, including size exclusion chromatography, ion exchange chromatography, reversed-phase high performance liquid chromatography and affinity chromatography. Sample fractions may be collected from each purification step, with those exhibiting biological activity in an appropriate assay being pooled and carried forward to the next purification step. This may result in the isolation of biologically pure MBF with or without its fusion partner.

Preferably, the presence of biologically pure MBF is detected by one or more of

a) migration as a single band of the appropriate size on SDS-PAGE gel chromatography;

b) N-terminal sequence analysis, or

c) mass spectroscopy.

The biologically pure MBF of the present invention is useful for a wide variety of purposes, including but not limited to

maintenance, growth or differentiation of animal or human cells in culture;

maintenance, growth or differentiation of more organised cellular structures, for example, skin, cartilage, tendon, ligament or bone;

coating a negatively-charged surface to promote cell adhesion, growth, migration, or activity, for example culture vessels for use in keratinocyte expansion to provide partial thickness skin grafts for burns patients, or coating of a surgical implant or a prosthesis;

enhancement of tissue remodelling and repair associated with trauma or manipulation, for example in the

treatment of wounds of all types, including burns, traumatic injuries, or surgical wounds;

as an orally active product for the prevention and/or treatment of impaired gut function;

facilitating tissue targeting following systemic administration, for example by localisation of the factor to bone matrix following intravenous injection; and

maintaining higher bioactive factor concentrations at the site of administration in order to effect a prolonged pharmacological action.

The MBF may be used in conjunction with or in combination with one or more other growth factors or therapeutic agents.

Accordingly, in a fourth aspect the present invention provides a composition for promoting adhesion, growth, migration, the prevention of apoptosis, or activity of cells in culture, comprising an effective amount of an MBF together with a pharmaceutically- or veterinarily-acceptable carrier.

In a preferred embodiment, this aspect of the invention provides a composition for the coating of a negatively-charged surface with an effective amount of MBF, thereby to promote adhesion, growth, migration or activity of cells.

In a fifth aspect the invention provides a method for promoting adhesion, growth, migration or activity of cells on a negatively-charged surface coated with an MBF, comprising the step of growing vertebrate cells in a culture medium over or on an appropriate surface pretreated with an MBF.

In both the fourth and fifth aspect of the invention the cells may be of vertebrate, preferably mammalian, or of insect origin.

In a sixth aspect, the invention provides a composition for the enhancement of tissue remodelling or tissue repair associated with tissue trauma or wound healing, comprising an effective amount of an MBF

formulated with a carrier such as an injectible, excipient, carrier, lotion, medicated body wash, dressing, liniment, toothpaste, mouthwash or powder.

In an alternative embodiment, this aspect of the invention provides a composition for alleviation of skin damage associated with ageing or with exposure to ultraviolet or ionizing radiation, comprising an effective amount of an MBF together with a cosmetically-acceptable carrier.

In a seventh aspect, the invention provides a composition for the prevention or treatment of a condition associated with impaired gut function, comprising an effective amount of an MBF formulated with a carrier suitable to produce an orally stable, bioactive enteral formulation.

In an eighth aspect, the invention provides a composition for the targeting or localisation of an MBF to cells or tissues, thereby to promote cell adhesion, growth, migration or activity *in vivo*, comprising an effective amount of MBF formulated in a pharmaceutically-acceptable carrier.

In a ninth aspect, the invention provides a method for the enhancement of tissue remodelling or tissue repair associated with tissue trauma or wound healing, comprising the step of administering an effective amount of an MBF to a subject in need of such treatment.

In a preferred embodiment, this aspect of the invention provides a method for the prevention or treatment of impaired gut function, comprising the step of administering an effective amount of an MBF to a subject in need of such treatment.

In a second preferred embodiment there is provided a method for the targeting and localisation of an MBF to cells or tissues, thereby to promote cell adhesion, growth, migration or activity *in vivo*, comprising the step of systemic or local administration of an MBF to a subject in need of such treatment.

It will be appreciated that in the therapeutic methods of the invention, the subject to be treated may be a human, or may be a domestic, companion or zoo animal.

The carrier to be used and the dose and route of administration will depend on the nature of the condition to be treated and the age and general health of the subject, and will be at the discretion of the attending physician or veterinarian. Suitable carriers and formulations are known in the art, for example by reference to Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania (1995). Suitable dosing regimens are established using methods standard in the art.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of SDS/PAGE analysis of the biologically pure MBF, as visualised by Novex Tricine gel chromatography.

Figure 2a shows dose-response curves of the extended forms of MBFs, L-MBF-1, L-MBF-2, L-MBF-3, L-MBF-4 and the extended form of IGF-I (L-IGF-I) for the competitive displacement of iodinated IGF-I from the IGF-I receptor isolated from human placental membranes.

Figure 2b shows dose-response curves of the cleaved forms of MBFs, MBF-1, MBF-2, MBF-3, MBF-4 and IGF-I for the competitive displacement of iodinated IGF-I from the IGF-I receptor isolated from human placental membranes.

Figure 3a shows dose-response curves of four extended forms of MBFs, L-MBF-1, L-MBF-2, L-MBF-3, L-MBF-4, and L-IGF-I in a protein synthesis assay using a myoblast cell line.

Figure 3b shows dose-response curves of four cleaved forms of MBFs, MBF-1, MBF-2, MBF-3, MBF-4 and IGF-I in a protein synthesis assay using a myoblast cell line.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be more fully described with reference to the accompanying non-limiting examples. It should be understood that the following description is illustrative only, and should not be taken in any way as a restriction on the generality of the invention. In particular, while the invention is specifically exemplified with reference to MBFs derived from IGF-I, it will be clearly understood that the methods described herein are applicable generally to the modification of polypeptide bioactive factors to generate MBFs, and to evaluation of the MBFs thus produced for biological activity. In particular, once the MBF is produced its testing for suitability for the purposes of the invention is a matter of routine.

Representative MBFs according to the invention were produced by introduction of heparin-binding motifs of other polypeptide bioactive factors into the sequence of human IGF-1. The methods used are described in detail below. It will be clearly understood that while the examples utilize the heparin-binding motif from bovine FGF-1, FGF-1 sequences from the human protein or from other species may also be used.

Sequence MBF-1 involves the deletion of the IGF-1 D-domain post Pro63 and its substitution with the heparin-binding motif Lys127 to Gln142 from bovine FGF-1, represented by the single letter code for amino acids as shown below. The introduced heparin-binding motif is shown in bold.

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEM YCAP**KKNGRSKLGPRTHFGQ**

(SEQ ID NO. 1)

Sequence MBF-2 contains all of the amino acids of native IGF-1, and Lys127 to Gln142 of FGF-1, through the insertion of the FGF-1 fragment Lys128 to Gly135 in between the residues Lys65 and Pro66 of IGF-1. This is followed by a second insertion of the FGF-1 segment Arg137 to Gln142 in between residues Pro66 and Ala67 of IGF-1. Overall, this results in the insertion of the entire FGF-1 fragment Lys128 to Gly142 which includes by Pro66 of IGF-1and is represented below by the single letter code for amino acids.

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEM YCAPLK**KNGRSKLGPRTHFGQ**AKSA

(SEQ ID NO. 2)

Sequence MBF-3 was constructed using a helical wheel optimised sequence element of FGF-1 (Lys127 to Gln142) that maximised the polarity of a theoretical helical model of the proposed IGF-1 variant. The optimisation analysis resulted in one glycine spacer amino acid being inserted in front of the FGF-1 sequence element and the substitution of glutamine for Lys133 and lysine for Leu134. The IGF-1 D-domain post Pro63 was deleted and this new FGF-1 segment was added and is represented below by the single letter code for amino acids.

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEM YCAP**GKKNGRSQKGPRTHFGQ**

(SEQ ID NO. 4)

Sequence MBF-4 most closely represents the native structure of the parent IGF-1 peptide. This variant has been designed utilising substitutions of amino acids and creates two recognised heparin-binding sequences; the

octapeptide XBBBXXBX (Asp-Lys-Arg-Gln-Leu-Glu-Lys-Tyr) and the hexapeptide XBBXB (Gly-Lys-Arg-Gly-Arg-Ser). These two structures are positioned either side of Cys61 and collectively constitute 7 changes in the A- and D-domains. This sequence represents an attempt to mimic heparin-binding structures seen in insulin-like growth factor binding proteins (IGFBPs) and heparin-binding EGF where the sequences surround a cysteine residue. The changes described are represented below by the single letter code for amino acids.

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCD**KRQLEK**
YCAP**GKRGR**SA

(SEQ ID NO. 5)

In every case, MBF cDNA constructs encode a fusion protein that results in a polypeptide containing the first 11 amino acids of porcine growth hormone, a linker of valine and asparagine (MFPAMPLSSLFVN) and the mutagenised hIGF-I sequence (MBF) and results in the expression of an extended form of MBF or Long MBF (L-MBF). These components permit the restriction digestion of the hIGF-I mutagenised sequence, the bacterial expression of the fusion protein and the hydroxylamine chemical cleavage of the leader sequence from the MBF sequence.

Example 1 Mutagenesis of a Nucleotide Sequence Encoding a Polypeptide Bioactive Factor (IGF-I) to Generate a Nucleotide Sequence Encoding an MBF

In order to perform site-directed mutagenesis on a nucleotide sequence encoding a polypeptide bioactive factor, a suitable plasmid cloning vector PTZ18 was obtained. The cDNA nucleotide sequence pMpGH(11)VN/IGF-I according to Example 5 of Australian Patent No. 633099, encoding pGH(1-11) joined via a potential hydroxylamine-

cleavable linkage N-terminal to IGF-I, was optimised for codon usage in bacteria, and subcloned into the PTZ18 plasmid cloning vector EcoR1/HindIII restriction site in 5'-3' orientation. The construct is hereinafter referred to as PTZ18/pGH(11)/hIGF-I. The optimisation involved generation, extraction and precipitation of PTZ18 plasmid cloning vector DNA and pMpGH(11)VN/IGF-I DNA, restriction enzyme digestions and ligations using conventional methods, for example as described in Molecular Cloning: A Laboratory Manual, Eds Sambrook, Fritsch and Maniatis (second edition), 1989; Pages 1.23-1.24, 1.62-1.68 respectively.

The correct nucleotide sequence was confirmed using the dideoxy-mediated chain termination (Sanger) method as described in Molecular Cloning, Pages 13.3-13.6.

Transformation of 200 µl of competent MV1190 bacterial cell suspension with 5 µl of PTZ18/pGH(11)/hIGF-I ligation reaction for the generation of quantities of PTZ18/pGH(11)/hIGF-I double stranded (ds) DNA to be used for restriction digests was carried out as described in Molecular Cloning, Pages 1.74-1.84. The remaining 5ul of ligation reaction was used to transform 200 µl of CJ236 bacterial cell suspension for the production of single stranded (ss) uracil containing DNA to be used for the production of replicative mutagenised ds DNA. Replicative ds mutagenised DNA was generated using site-directed mutagenesis. In each case two oligonucleotide primers were employed to insert the changes necessary to create a nucleotide sequence encoding an MBF. The complete mutagenesis in each case employed the prime (') oligonucleotide for the first reaction, while the second reaction employed the double prime('') oligonucleotide and used the methods described in Molecular Cloning, Pages 15.74-15.79 and 15.63-15.65. For example, to complete the mutagenesis of MBF-2, IGFS-2' was employed to achieve the first round of mutagenic changes and IGFS-2'' was employed to achieve the second round of mutagenic changes.

Oligonucleotide IGFS-2' (54 mer)
5'-TGCGCTCCGCTGAAAAAAAACGGTCGTTCTAAACTGGGCCCGGCTAAATCTGCT-3'
(SEQ ID NO. 6)

Oligonucleotide primer IGFS-2'' (48 mer)
5'-TCTAAACTGGGTCCGCGTACCCACTTCGGCCAGGCTAAATCTGCTTGA-3'
(SEQ ID NO. 7)

Transformants carrying the correct ds MBF-2 DNA, hereinafter referred to as PTZ18/pGH(11)/MBF-2, were determined by the dideoxy-mediated chain termination (Sanger) method, and used to generate quantities of PTZ18/pGH(11)/MBF-2 DNA.

A number of other PTZ18/pGH(11)/MBF analogue constructs were generated using different oligonucleotide primers and identical molecular biology techniques (see examples).

Oligonucleotide primers IGFS-1' and IGFS-1'' encoding MBF-1:

Oligonucleotide primer IGFS-1' (54 mer)
5'-ATGTACTGCGCTCCGAAAAAAAACGGTCGTTCTAAACTGCTGAAACCGGCTAAA-3'
(SEQ ID NO. 8)

Oligonucleotide primer IGFS-1'' (54 mer)
5'-GGTCGTTCTAAACTGGGCCCGCGTACCCACTTCGGTCAGTGATGATGC**AA**GCTT-3'
(SEQ ID NO. 9)

Oligonucleotide primers IGFS-3' and IGFS-3'' encoding MBF-3:

Oligonucleotide primer IGFS-3' (57 mer)
5'-ATGTACTGCGCTCCGGGTAAAAAAAACGGCCGTTCTCAGAAACTGAAACCGGCTAAA-3'
(SEQ ID NO. 10)

Oligonucleotide primer IGFS-3'' (54 mer)
5'-GGTCGTTCTCAGAAAGGCCCGCGTACCCACTTCGGTCAGTGATGATGCAAGCTT-3'
(SEQ ID NO. 11)

Oligonucleotide primers IGFS-4' and IGFS-4'' encoding MBF-4:

Oligonucleotide primer IGFS-4' (48 mer)
5'-TTCCGTTCTTGCGACAAACGTCAGCTGGAAAAATACTGCGCTCCGCTG-3'
(SEQ ID NO. 12)

Oligonucleotide primer IGFS-4'' (45 mer)
5'-AAATACTGCGCTCCGGGTAAACGTGGCCGTTCTGCTTGATGATGC-3'
(SEQ ID NO. 13)

Example 2 Subcloning the Nucleotide Sequence Encoding an MBF from PTZ18/pGH(11)/MBF into Expression Vector pGHXSC.4

pGHXSC.4 already contains within its DNA the nucleotide sequence encoding pGH(11). The nucleotide sequence encoding only the MBF was excised from PTZ18/pGH(11)/MBF DNA using restriction enzymes HpaI/HindIII. This MBF nucleotide sequence was subcloned into expression vector pGHXSC.4, using the techniques described in Example 1. The thus-formed expression vector construct is hereinafter referred to as pGHXSC.4/MBF.

ds DNA sequencing as described in Example 1 was again employed to confirm the expected nucleotide sequence of the MBF fusion protein in the expression vector.

Example 3 Transformation of JM101 Bacterial Cells with the Expression Vector pGHXSC.4/MBF Containing the Nucleotide Sequence Encoding an MBF

To facilitate the expression of the MBF fusion protein, pGHXSC.4/MBF (Example 2) was transformed by methods outlined in Example 1 into a suitable host cell, in this case $lacI^q$ JM101 cells.

Transformants containing pGHXSC.4/MBF successfully grew on agarose plates containing ampicillin.

Example 4 Induction of JM101 Bacterial Cells Transformed with pGHXSC.4/MBF to Determine Cell Clones Expressing MBF Fusion Proteins as IBs

To confirm that the cells were expressing the fusion protein as inclusion bodies (IBs), inductions of single clones were undertaken. Single pGHXSC.4/MBF transformed colonies (Example 3) were inoculated into Luria Bertani (LB) medium and cultured overnight. An aliquot from each of these cultures was transferred to a fresh sample of LB medium and incubated at 37°C until an absorbance at 600 nm (A_{600}) of 0.8-1.0 was reached. At this time an aliquot was taken from each culture and reserved. To the remainder of the culture was added isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, to induce the cells into the production of the MBF fusion protein encoded within pGHXSC.4/MBF. Following further incubation, both the reserved culture and the induced culture were centrifuged to pellet the cell, and following removal of the supernatant the cells were treated with 2% β-mercaptoethanol/10% SDS to lyse the cells and denature the protein.

Pre-induced cultures were compared directly with post-induced cultures, using SDS/PAGE gel chromatography, 8-25% gradient Phast gel (Pharmacia) to determine MBF fusion protein expressing clones and to confirm the estimated size of the MBF.

Example 5 Production of MBF Fusion Protein Using Cells Expressing MBF Fusion Protein as Inclusion Bodies (IBs)

Clones identified as expressing the fusion protein in Example 4 were used in a scale-up process to produce appropriate quantities of the fusion protein for *in vitro* and *in vivo* experiments.

Four 2 litre Applicon fermenters were employed, each containing 1L of minimal medium and inoculated with an

aliquot of a culture, established with clones expressing MBF fusion proteins, growing in log phase. Inoculated fermentation cultures were incubated at 37°C overnight. At an absorbance at 600 nm (A_{600}) of between 4-6, IPTG was added to the cultures to a final concentration of 0.2 mM and the cells further incubated until an A_{600} of approximately 15-20 was reached, after which the fermentation suspension was subjected to a number of passes through a homogeniser. This process disrupted the cells, facilitating the further isolation and purification of IBs by three centrifugation and washing steps, using 30 mM NaCl/10 mM KH_2PO_4 washing buffer.

For pGHXSC.4/MBF-2 this process yielded a wet IB pellet of 8.2 grams, which was stored at -20°C.

Example 6 Dissolution, Refolding, and Cleavage of MBF Fusion Protein Produced in Inclusion Bodies, Purification of Biologically Pure MBF

The wet IB pellet containing the MBF-2 fusion protein from Example 5 was solubilised, desalted and refolded by conventional methods. The MBF fusion protein was then isolated and cleaved, followed by chromatographic steps to yield a biologically pure MBF, employing known methods. These processes included, in sequence:

1) dissolution of IBs in buffer (8 M urea, 0.1 M Tris, 40 mM glycine, 0.5 mM $ZnCl_2$ and 40 mM dithiolthreitol (DTT) pH 9.1), centrifugation and filtration (1 µm gradient Whatman filter) to remove particulate contaminants and desalting into 8 M urea, 0.1 M Tris, 40 mM glycine, 0.5 mM $ZnCl_2$ and 1.6 mM dithiothreitol pH 9.1 by size exclusion chromatography on Cellufine GCL-1000m;

2) a 330 ml pool of buffer containing 104.1 mg of fusion protein was reconstituted to 1.320 L in 2.5 M urea, 40 mM glycine, 0.1 M Tris, 0.4 mM DTT and 10 mM ethylenediaminetetra-acetic acid (EDTA) pH 9.0; refolded over 120 minutes with the addition of 0.12ml.L^{-1}

of oxidised β-mercaptoethanol and re-acidified to pH 2.5 with concentrated HCl;

3) cation exchange on a SP Sepharose Fast Flow (FFS) matrix, eluting the protein with 8 M urea, 50 mM Ammonium acetate and 1M NaCl pH 4.8;

4) a 160 ml (FFS) pool of protein containing 97.3 mg was divided with 20% reserved, after which 80% was reconstituted to 2 M urea, 0.1 M Tris, 1 M NH_2OH and 1 mM EDTA pH 8.65 followed by cleavage (see below) for 24 hrs at 40°C;

5) buffer exchange of both the reserved material and cleaved material was achieved by C18 matrix fast performance liquid chromatography (FPLC) using an XK50/20 column (Pharmacia), washing with 0.1% trifluroacetic acid (TFA) and eluting with 80% acetonitrile/0.08% TFA, followed by

6) final desalting and purification by high performance liquid chromatography (HPLC) on a C4 matrix PrepPak column (Waters) washing with 0.1% TFA and eluting with an 80% acetonitrile/0.08% TFA gradient at 0.1% per minute.

Cleavage of the MBF fusion protein yields a fusion partner and MBF, and may be employed to further potentiate the bioactivity of the MBF if necessary. Thus two MBFs may be derived from the one MBF fusion protein, an extended form of MBF having the first 11 amino acids from porcine growth hormone (pGH(1-11)) N-terminally linked to the MBF amino acid sequence (L-MBF), and a cleaved form not having pGH(1-11).

Dissolution, refolding and cleavage (if used) of MBF fusion protein derived from the pGHXSC.4/MBF expression vector construct in the foregoing manner yielded material that ran as a single band following SDS/PAGE Novex Tricine gel chromatography, as shown in Figure 1. This material was represented as the major species following electrospray mass analysis, and was observed at the calculated theoretical mass.

Example 7 The Affinity of Biologically Pure MBF for Heparin as Measured by Heparin Affinity Chromatography

10 µg aliquots of biologically pure MBF-2 and L-MBF-2 from Example 6 were reconstituted in 10 µl of 10 mM HCl, taken up into a final volume of 100 µl and loaded in 10 mM Tris pH 7.0 on to a Pharmacia heparin-Sepharose CL6B affinity column connected to a FPLC and eluted with a linear gradient (0 M NaCl-1M NaCl) of 10 mM Tris/1 M NaCl pH 7.0 over 50 minutes. A 10 µg aliquot of authentic IGF-I and pGH(1-11) IGF-I (L-IGF-I) was also loaded and eluted using the same conditions.

The affinity of cleaved MBFs was such that they required salt concentrations of between 0.26 M and 0.33 M to elute them from the heparin matrix. L-MBFs required salt concentrations of between 0.24 M and 0.32 M, whereas IGF-I and L-IGF-I eluted at salt concentrations of 0.12 M and 0.11 M respectively.

Similarly, 10 µg aliquots of MBFs, L-MBFs, IGF-I and L-IGF-I were prepared as described and loaded in 10 mM Tris pH 7.0 on to a Progel TSK Heparin affinity column connected to a HPLC. Proteins were again eluted with the previously described salt gradient.

MBFs required salt concentrations of between 0.53 M and 0.59 M, while L-MBFs required salt concentration of between 0.56 M and 0.89 M to elute them from this column. IGF-I and L-IGF-I eluted at salt concentrations of 0.28 M and 0.29 M respectively.

This example shows that MBFs do indeed bind more avidly to negatively charged materials as exemplified by two types of heparin-affinity columns.

Example 8 *In vitro* Binding Affinity of Biologically Pure MBF for the IGF-I Receptor Isolated from Human Placental Membranes

The biologically pure MBFs derived in Example 6 had affinities for the IGF-I receptor which ranged from equipotent to three fold lower than authentic IGF-I or uncleaved long (L-IGF-I). IGF type I receptors isolated from human placental membranes (Cuatrecasas, P., J. Biol. Chem., 1972 247 1980-1991) were incubated with iodinated hIGF-I or L-hIGF-I in the presence of increasing concentrations of MBFs or L-MBFs (0.01 pmol to 100 pmol). The affinity of the MBFs or L-MBFs was measured by the competitive displacement of iodinated hIGF-I or L-hIGF-I from the receptors and the results expressed as percentages of iodinated IGF-I remaining bound to the IGF-I receptor. The results are shown in Figures 2a and 2b.

Example 9 *In vitro* Stimulation of Protein Synthesis by Biologically Pure MBF in a Myoblast Cell Line

The biologically pure MBFs from Example 6 stimulated the production of proteins by rat L6 myoblasts in serum-free medium (Francis *et al*, Biochem. J., 1985 233 207). The ability of increasing concentrations of MBFs, ranging from 1 ng/ml to 1 µg/ml, to stimulate protein synthesis in rat L6 myoblasts was measured, and compared with the ability of both commercially-derived IGF-I and L-IGF-I (GroPep) to stimulate protein synthesis in rat L6 myoblasts. Results are expressed as the percentage stimulation of protein synthesis above that observed in growth factor-free or serum free medium, and shown in Figures 3a and 3b.

Example 10 Characteristics of Binding of Iodinated Biologically Pure MBF to Negatively-Charged Surfaces

The biologically pure MBFs from Example 6 were iodinated by conventional methods, and shown to have the

ability to bind to two negatively-charged surfaces. Iodinated L-MBFs and reference peptides (10,000 counts per minute cpm/well), when incubated overnight at 4°C in 24-well polyanionic tissue culture plastic plates in the presence of 1 ml of 1.0% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS) and then washed twice using 1 ml of 1.0% BSA/PBS, demonstrated an increase of approximately 6 to 15-fold in their ability to remain bound to the substrate, compared to that of iodinated L-IGF-I (10,000 cpm/well) incubated under the same conditions.

The results, shown in Table 1, are expressed as the number of counts per minute (cpm) retained by the iodinated polypeptide bioactive factor following the washing steps.

Table 1

Radioactive counts per minute (cpm) retained on tissue culture plastic

Iodinated polypeptide bioactive factor	**Counts per minute retained (means ± sem)**
FGF-2	1911.6 ± 16.7
IGF-II	967.6 ± 29.3
L-IGF-I	291.3 ± 34.6
L-MBF-1	4758.3 ± 107.4
L-MBF-2	4523.1 ± 89.6
L-MBF-3	4976.3 ± 122.5
L-MBF-4	1970.2 ± 77.4

Similarly, biologically pure, iodinated L-MBFs (10,000 cpm/well) when incubated overnight at 4°C in 1.0% BSA/PBS on HaCat epithelial cell-derived matrix in 24-well plates (Jones *et al*, J. Cell Biol., 1993 121 679) and then washed twice with 1.0% BSA/PBS exhibited increases of approximately up to 5-fold in their ability to remain

bound to the matrix when compared with iodinated L-IGF-I (10,000 cpm/ml), as shown in Table 2.

Table 2

Radioactive counts per minute (cpm) retained on HaCaT cell derived matrix

Iodinated polypeptide bioactive factor	**Counts per minute retained (means ± sem)**
FGF-2	1697.4 ± 73.6
IGF-II	2168.6 ± 201.3
L-IGF-I	80.9 ± 4.7
L-MBF-1	370.6 ± 29.7
L-MBF-2	406.2 ± 16.8
L-MBF-3	398.7 ± 14.1
L-MBF-4	78.8 ± 7.9

Example 11 *In vitro* Stimulation of Protein Synthesis in an Epithelial Cell Line by Biologically Pure MBF Bound to a Negatively-Charged Surface

The four extended forms of biologically pure MBFs from Example 6, which were shown to stimulate protein synthesis in a myoblast cell line (Example 9) and to have the ability to bind to negatively-charged surfaces (Example 10), were compared with authentic IGF-II, IGF-I and L-IGF-I for their ability to stimulate protein synthesis in an epithelial cell line following pre-incubation and retention on two negatively-charged surfaces. MBFs, IGF-II, IGF-I and L-IGF-I were incubated overnight at 4°C in 0.5 ml of 1.0% BSA/PBS at concentrations of 2 ng/ml, 20 ng/ml and 200 ng/ml in 24-well tissue culture plates which were either untreated or coated with HaCat epithelial cell-derived matrix, and then washed twice using 1 ml of 1.0% BSA/PBS, as described in Example 10.

HaCat epithelial cells were serum starved for 2 hours, harvested and resuspended in serum-free medium containing 1 μCi/ml H^3 leucine, after which they were seeded on to MBF, IGF-II, IGF-I or L-IGF-I pre-incubated and washed wells at a density of 2.85 x 10^5 cells/well, and incubated for a further 18 hrs at 37°C. Wells were washed twice with 1 ml of cold Hanks balanced salt solution, followed by a single wash in 0.5 ml of cold 5% trichloroacetic acid , after which wells were washed with 0.5 ml of cold reverse osmosis quality water. Finally 0.25 ml of 0.1% Triton X-100/0.5 M NaOH was added to each well and shaken for 30 mins. The Triton X-100/NaOH solution from each well was then assayed for beta-emitting radiation, indicative of ^{3}H-leucine which had been incorporated into proteins produced by the cells during the 18 hr incubation at 37°C. The results are shown in Tables 3 and 4.

Bound MBFs showed dose-dependent stimulation of protein synthesis in HaCat epithelial cells, between 1.5- and 2-fold greater than that exhibited by IGF-II, IGF-I or L-IGF-I in the untreated negatively-charged plastic tissue culture vessel. For matrix-bound MBFs, stimulation of protein synthesis in HaCat epithelial cells was approximately 30% above that induced by IGF-I and L-IGF-I, and was observed only at the highest concentration of 200 ng/ml (Table 3 and 4).

Table 3

Stimulation of protein synthesis in HaCaT cells seeded onto polypeptide bioactive factor pre-treated tissue culture plastic.

Polypeptide bioactive factor	**2 ng/ml**	**20 ng/ml**	**200 ng/ml**
FGF-2	112.3 ± 7.5	102.6 ± 4.7	130.4 ± 7.0
IGF-II	117 ± 13	109.8 ± 5.1	134.1 ± 2.7
L-IGF-I	114 ± 6.3	109.8 ± 13.6	132.3 ± 6.3
L-MBF-1	131.7 ± 3.7	175.4 ± 9.4	213.0 ± 10.6
L-MBF-2	131.2 ± 5.2	159.9 ± 10.2	220.5 ± 9.6
L-MBF-3	133.2 ±10.2	153.7 ± 13.2	226.8 ± 5.6
L-MBF-4	143.3 ± 5.6	165.6 ± 6.6	214.6 ± 5.2

Table 4

Stimulation of protein synthesis in HaCaT cells seeded onto polypeptide bioactive factor pre-treated HaCaT cell derived matrix

Polypeptide bioactive factor	**2 ng/ml**	**20 ng/ml**	**200 ng/ml**
FGF-2	82.4 ± 3.3	110.5 ± 13.0	110.9 ± 8.3
IGF-II	96.1 ± 6.1	98.6 ± 10.0	107.3 ± 2.2
L-IGF-I	102.3 ± 3.8	103.3 ± 8.7	113.1 ± 7.3
L-MBF-1	101.3 ± 4.9	100.4 ± 7.3	130.9 ± 6.4
L-MBF-2	98.3 ± 4.2	102 ± 10.5	133.2 ± 3.6
L-MBF-3	103.8 ± 7.9	102.5 ± 9.9	135.2 ± 7.2
L-MBF-4	103.7 ± 5.9	114.1 ± 17.4	124.1 ± 11.8

Example 12 *In vitro* Binding of Pure MBF-2 and L-MBF-2 to Titanium Screws

Biologically pure MBF-2 and L-MBF-2 from Example 6 were iodinated by conventional methods and shown to have an increased ability to bind to titanium screws, compared to iodinated IGF-I. Iodinated MBF-2 and L-MBF-2 were diluted into Dulbecco's modified minimal medium (DMEM) (10,000 counts per minute/ml).Titanium screws were incubated in the presence of 1ml of iodinated MBF or IGF-I solution (10,000 cpm/ml) overnight at 4°C in 24-well tissue culture plastic plates. The medium was removed, and the screws were each washed twice with 1 ml of cold DMEM. The washing medium and the screws were analysed for the presence of the iodinated MBF or IGF-I species.

The MBFs demonstrated between 2.5 and 4.5-fold increases in their ability to remain bound to the titanium screws when compared to iodinated IGF-I. The results, shown in Table 5, are expressed as the number of counts per minute (cpm) retained on the screws following the washing steps.

Table 5

Radioactive Counts
Remaining Associated with Titanium Screws
Following Two DMEM Washes (n=3)

Treatment	cpm/SCREW	cpm/SCREW (mean ± sem)
IGF-I	97.9	
IGF-I	87.3	83.8 ± 11.4
IGF-I	66.1	
MBF-2	337.4	
MBF-2	416.9	373.4 ± 28.5
MBF-2	365.8	
L-MBF-2	211.5	
L-MBF-2	189.7	209.3 ± 13.1
L-MBF-2	226.7	

Example 13 *In vitro* Retention of MBF-2, L-MBF-2 and IGF-I in Fibrin Gels

Biologically pure MBF-2 and L-MBF-2 from Example 6 were iodinated by conventional methods, and shown to have an increased ability to remain bound within fibrin gels or clots, compared to iodinated IGF-I. 50 µl of 0.4% fibrinogen containing 10,000 cpm of iodinated MBF or IGF-I was combined with 5 µl of 0.02% thrombin in 24-well tissue culture plastic plates to form a fibrin clot or gel. 1 ml of DMEM was added to each well to cover the clots and incubated at 4°C for 24 hours, after which the medium was collected and replaced with fresh DMEM. This process of medium collection and replacement continued for 48 hours. Collected medium was analysed for the presence of iodinated MBF or IGF-I.

The MBFs demonstrated up to 50% increases in retention within the fibrin gels as compared to IGF-1 at all time points, as shown in Table 6.

Table 6

Retention of Radioactive Counts (Iodinated Peptide) Within Fibrin Gels over 48 hours

Sample	% Counts Retained	
	24 hours	48 hours
IGF-I	19.4	14.9
MBF-2	24.5	19.4
L-MBF-2	26.2	20.9

Example 14 Adsorption of MBF-2, L-MBF-2 and IGF-I on to Polyanionic Tissue Culture Plastic

Solutions of MBF-2, L-MBF-2 and IGF-I were prepared to a final concentration of 100 ng/ml in DMEM. 1 ml of each solution was applied to the first well of separate 24-well tissue culture plastic plates, and incubated at room temperature for 15 minutes. Following this incubation period the 1 ml solutions were transferred to the second well of each 24-well plate, and incubated at room temperature for another 15 minutes. This process was repeated for a total of 18 out of the total 24-wells in each plate.

Following the sequential coating of 18 wells by either a 1 ml solution of MBF-2, L-MBF-2 or IGF-I, the wells were washed twice with 1 ml of DMEM, and air dried within a laminar flow cabinet. 1 ml of DMEM containing 2.5×10^5 HaCat epithelial cells and 1 µCi of tritiated leucine was added to each well and incubated at 37°C for 18 hours. Wells were washed twice with 1 ml each of Hank's balanced salt solution, twice with 1 ml each of 5% trichloroacetic acid (TCA), and once with 2 ml of Milli-Q water. 1 ml of 0.5M sodium hydroxide/0.1% triton X-100 was added to each well and incubated at room temperature for at least 30 minutes, with shaking. 100 µl samples from each

well were transferred to scintillation vials, 2 ml of scintillation fluid was added to each vial, and mixed well with shaking. Samples were analysed for the presence of β-emitting tritiated leucine incorporated into newly-synthesized protein in response to the MBF or IGF-I bound to the plastic surface.

Results are expressed as a percentage of protein synthesis compared to a growth factor-free control, and are shown in Table 7. 1 ml solutions of biologically pure MBF-2 and L-MBF-2 (100 ng/ml) from Example 6 were able to be used repeatedly (at least 18 applications) to coat tissue culture plastic surfaces, with 5-8 fold stimulation of protein synthesis in HaCat cells grown on these surfaces. In contrast, the repeated coating of tissue culture plastic surfaces with IGF-1 solution resulted in a lower stimulation of protein synthesis by the HaCat cells, which returned to baseline after 13 applications.

Table 7

Stimulation of Protein Synthesis in HaCat Cells Grown on to 24-well Tissue Culture Plates Treated with MBF-2, L-MBF-2 or IGF-I, Expressed as a Percentage of a Growth Factor-Free Control

Diluted Series	IGF-1	MBF-2	L-MBF-2
1	334.4	611.9	876.8
2	442.3	578.7	751.3
3	400.7	560	705.5
4	267	608.2	708.9
5	293.3	592.6	742
6	243.7	660.1	814.3
7	234.4	643.1	758.7
8	203.5	642.6	830.1
9	192.9	613.4	706.9
10	202.3	603.5	712.8
11	230.1	549.1	655.9
12	178.1	539.3	706.2
13	86.4	550.9	632.4
14	128.1	533.7	676.2
15	184.7	566.5	686
16	122.2	572.7	675.1
17	106	615.3	774
18	97.9	628.2	717.3

Example 15 *In vivo* Tissue Distribution of Systemically Administered Iodinated MBF-2, L-MBF-2 Compared to IGF-I

Biologically pure MBF-2 and L-MBF-2 from Example 6 iodinated by conventional methods and injected via jugular catheter into male rats appeared to localise preferentially to a variety of tissues, when compared to similarly iodinated and administered IGF-I.

Male Sprague Dawley rats (118-130 grams) were administered 1 x 10^7 cpm of either MBF-2, L-MBF-2 or IGF-I, and decapitated at either 1 minute or 15 minutes, after which samples of blood and the gut, left hind limb, pelt, heart, liver, spleen, lungs, adrenals, kidneys and thymus were frozen in liquid nitrogen. Samples were thawed before being homogenised in 5 volumes (w/v) of 10% TCA and analysed for the presence of TCA precipitable iodinated MBF-2, L-MBF-2 or IGF-I.

Results for each tissue were expressed as cpm/gram of tissue and normalised compared to cpm in the plasma of each respective rat, to produce a ratio of between 0 and 1 correlating to cpm/gram of tissue. Table 8 shows the tissues where preferential localisation of MBFs occurs compared to IGF-I.

Table 8

Tissue localisation of iodinated MBF-2 and L-MBF-2 Compared to IGF-I

(data normalised to plasma counts)

(n=2)

Tr atment/Kill Time	Skin	Muscle	Liver	Tendon	Bone	Stomach	Small Intestine
IGF-I at 1 minute	0.025	0.016	0.17	0.017	0.075	0.11	0.09
	0.024	0.016	0.16	0.020	0.075	0.17	0.08
IGF-I at 15 minutes	0.060	0.022	0.060	0.039	0.12	0.19	0.12
	0.055	0.018	0.055	0.037	0.12	0.20	0.10
L-MBF-2 at 1 minute	0.053	0.037	0.37	0.035	0.18	0.33	0.25
	0.063	0.037	0.34	0.050	0.14	0.27	0.15
L-MBF-2 at 15 mins	0.088	0.037	0.27	0.075	0.20	0.26	0.21
	0.063	0.026	0.25	0.042	0.21	0.26	0.25

Example 16 Effect of L-MBF-2 on Growth and Cellular Activity of CHO Cells Grown on Polypropylene Discs

30 mls of 0.9% saline, 2 grams of polypropylene discs were placed in four 100 ml spinner flasks and autoclaved. The saline in two of the spinner flasks was removed, replaced with 30 mls of DMEM containing 100 ng/ml L-MBF-2, and all four flasks stored overnight at 4°C. The polypropylene discs in all four-spinner flasks were washed twice with 50 mls of DMEM and seeded with 1 x 10^7 CHO cells expressing marmoset chorionic gonadotrophin in 50 mls of PF-CHO protein-free medium. The spinner flasks were allowed to stand for 1 hour, after which they were incubated at 5% CO_2, 37°C on a multiple magnetic stirrer. 1 ml sub-samples of medium were collected from each spinner flask at 2 hrs, 4 hrs, 16 hrs, 20 hrs, 24 hrs, 48 hrs, 72 hrs and frozen immediately. The sub-samples were analysed for glucose concentration and marmoset chorionic gonadotrophin (ELISA) as indicators of cellular growth and/or activity.

Results at each sub-sample time are expressed as glucose concentrations (mM), as shown in Table 9, and as protein (chorionic gonadotrophin) production, as measured by colorimetric ELISA assay at 650 nm, shown in Table 10. These results showed that CHO cells grown on polypropylene discs treated with biologically pure L-MBF-2 from example 6 contained within 100 ml spinner flasks were more active, as indicated by glucose consumption and marginally increased protein production, than identical cells grown on untreated polypropylene discs.

Table 9

Glucose Consumption over the 72 hour Period

Sample	Glucose Concentration (mM)			
	Treated Discs		Untreated Discs	
2 hours	21.8	21.8	21.8	21.8
4 hours	20.8	20.7	21.6	21.6
16 hours	19.3	19.2	21.1	21.0
20 hours	18.7	18.9	20.9	20.7
24 hours	15.9	15.7	20.7	20.3
48 hours	13.9	13.6	16.6	16.5
72 hours	9.8	8.5	11.5	12.1

Table 10

Marmoset Chorionic Gonadotrophin in Conditioned Medium as Measured by ELISA

Sample	Absorbance (650 nm)			
	Treated Discs		Untreated Discs	
2 hours (1:4 dilution)	0.43	0.4	0.36	0.4
4 hours (1:4 dilution)	0.44	0.4	0.37	0.4
16 hours (1:8 dilution)	0.41	0.41	0.38	0.37
20 hours (1:8 dilution)	0.46	0.44	0.39	0.39
24 hours (1:8 dilution)	0.48	0.51	0.45	0.47

Example 17 The Protective Effects of MBF Coated Tissue Culture Plastic Against Apoptosis induced by Serum Deprivation or Camptothecin

The tissue culture wells were each incubated with 1 ml of DMEM containing 100 ng/ml of MBF-2 or L-MBF-2 for 18 hours at 4°C. Following the 18 hour incubation all wells were washed twice with 1 ml of DMEM, and allowed to air dry in a laminar flow cabinet. An identical number (0.2×10^5) of MCF7 mammary tumour cells was seeded on to and grown on the MBF-treated and untreated 24-well tissue culture plastic plates. The number of cells on the respective plates induced into apoptosis by either serum deprivation or the addition of camptothecin was indicated by the detection of propidium iodide staining.

Results are expressed as the number of cells per field staining positive for propidium iodide. Four fields in each of two wells were assessed for each treatment (n=8), and the results are shown in Table 11. This experiment showed that biologically pure MBF-2 and L-MBF-2 from Example 6 coated on 24-well tissue culture plastic plates had a protective effect against serum deprivation or drug-induced apoptosis in a mammary tumour cell-line.

Table 11

Number of cells per field induced into apoptosis

Sample	Control wells (mean, n=8)	MBF-2 treated wells (mean, n=8)	Control wells (mean, n=8)	L-MBF-2 treated Wells (mean, n=8)
Serum-deprived	102 ± 18.8	53 ± 3.7	108 ± 14.7	48 ± 3.7
camptothecin (1μM)	76 ± 12.9	38 ± 4.7	84 ± 11.1	36 ± 5.6

Example 18 The Stimulation of Protein Synthesis in HaCat Epithelial Cells Seeded on to Various Biological Substrates Pre-Treated with MBF-2, L-MBF-2 or IGF-I

Preparation of substrates

24-well tissue culture plastic plates were coated with 1 ml of poly-L-lysine solution (0.1%) for 10 minutes, after which each well was washed twice with sterile Milli-Q water and allowed to air dry for at least 2 hours. Heparin, dextran sulphate and chondroitin sulphate A were dissolved in sterile water (100 µg/ml), 1 ml of each solution added to respective wells and incubated for 18 hours at 4°C. The wells were washed twice with 1 ml of sterile Milli-Q water, and allowed to air dry inside a laminar flow cabinet for at least 1 hour. These plates were stored at 4ºC until pre-treatment with MBFs or IGF-I.

Rat tail collagen was prepared according to a conventional method and 250 µl of the stock collagen solution added to respective poly-L-lysine coated wells. After a 5 minute incubation inside a laminar flow cabinet the collagen solution was aspirated, leaving only a thin film of collagen remaining, and the collagen-coated wells air dried for at least 1 hour, after which they were stored at 4°C until pre-treatment with MBFs or IGF-I.

Fibronectin and laminin coated plates were purchased from Falcon, and pre-treated with MBFs or IGF-I as supplied.

Pre-treatment of substrates

Biologically pure MBF-2 and L-MBF-2 from Example 6 and receptor grade IGF-I were dissolved in DMEM at 100 ng/ml. 1 ml of these solutions was added to the respective pre-prepared wells and incubated for 4 hours at 4ºC. All wells were washed twice with 1 ml of cold DMEM and stored at 4°C prior to inoculation with cells.

Seeding with cells

HaCat cells were grown in the absence of serum for 4 hours and harvested. Cells were counted and resuspended into DMEM containing 1 µCi/ml of tritiated leucine at 2 x 10^5 cells/ml. 1 ml of cell suspension containing radioactive tracer was added to each of the pre-prepared and pre-treated wells, and incubated at 37°C for 18 hours.

Harvesting

The wells were washed twice with 1 ml of cold Hank's balanced salt solution, twice with 1 ml of cold 5% trichloroacetic acid, and once with 2 ml of cold Milli-Q water. 1 ml of 0.1% Triton X-100/0.5M NaOH was added to each well and incubated at room temperature for 30 minutes with shaking. 100 µl sub-samples from each well were transferred to scintillation vials, 2 ml of scintillation fluid added to each vial and mixed well with shaking. Sub-samples were assayed for the presence of newly synthesized tritiated-leucine containing protein.

Results are expressed as the percentage of protein synthesis stimulation compared to a growth factor-free control, and are shown in Table 12. This study showed that HaCat cells grown on 24-well tissue culture plastic plates coated with heparin, dextran sulphate, chondroitin sulphate A, fibronectin, laminin and rat-tail collagen respectively that had been pre-treated with MBF-2 or L-MBF-2 all exhibited increased stimulations of protein synthesis, compared with the same substrates pre-treated with IGF-I. No effect was found with poly-L-lysine coated plates. Similar results were obtained in a second experiment.

Table 12

Experiment 1

Stimulation of Protein Synthesis, Expressed as Percentage of Growth Factor-Free Control (n=3)

Substrate

Growth Factor	Plastic	Heparin	Dextran sulphate	Chondroitin sulphate A	Collagen	Poly-L-lysine	Fibronectin	Laminin
MBF-2	304.5	213.3	213.1	218.7	253.1	105.8	160.3	162.8
L-MBF-2	303.7	210.7	213.6	218.2	260.5	103.7	162.0	160.4
IGF-I	109.9	106.7	109.3	108.8	135.4	111.5	99.8	96.3

Experiment 2

Stimulation of Protein Synthesis Expressed as Percentage of Growth Factor-Free Control (n=3)

Substrates

Growth Factor	Plastic	Heparin	Dextran sulphate	Chondroitin sulphate A	Collagen	Poly-L-lysine	Fibronectin	Laminin
MBF-2	319.7	218.0	215.2	220.3	264.7	103.9	164.9	173.7
L-MBF-2	344.2	216.7	221.5	222.1	250.3	105.8	167.6	169.4
IGF-I	114.3	109.4	114.0	104.5	135.8	104.8	103.2	97.6

Example 19 The Stimulation of Tenocyte Cell Migration from Tendon Biopsies by MBF-2

The migration of fibroblast type cells (tenocytes) from circular tendon biopsies into fibrin clots was stimulated by biologically pure MBF-2 from Example 6. 2 mm diameter tendon biopsies were taken from chicken toe flexor tendons and embedded into fibrin clots. The fibrin clots containing tendon biopsies were incubated in medium containing MBF-2 (500 ng/ml) + 5% fetal bovine serum (FBS), IGF-I (500 ng/ml) + 5% FBS or in 5% FBS alone and incubated at 5% CO_2, 37ºC for 4 days. The migration distances of cells were measured four times each day at three-hourly intervals using phase-contrast light microscopy (4X magnification) and the mean calculated for each treatment.

Results were expressed as the migration distance in millimetres at each of the four days, and are shown in Table 13. This experiment demonstrated greater migration distances with MBF-2 and IGF-I above control (5%FBS) cultures. MBF-2 was slightly more active than IGF-I at the earlier time points.

Table 13

Migration Distances (mm) of Tenocytes from their Biopsy Interface

	IGF-1 (500 ng/ml) + 5% FBS	**MBF-2 (500 ng/ml) + 5% FBS**	**5% Fetal bovine serum**
Day 1	0.8 ± 0.1	1.0 ± 0.1	0.4 ± 0.1
Day 2	2.7 ± 0.4	3.4 ± 0.3	1.6 ± 0.2
Day 3	5.3 ± 0.6	5.7 ± 0.5	3.5 ± 0.4
Day 4	7.8 ± 0.8	7.8 ± 0.4	5.7 ± 0.6

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding,

various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

CLAIMS

1. A recombinant matrix binding factor (MBF), comprising a polypeptide bioactive factor in which the naturally-occurring amino acid sequence of the factor has been modified to introduce one or more amino acid substitutions, deletions and/or additions which increase the affinity of the polypeptide bioactive factor for a negatively-charged surface.

2. An MBF according to Claim 1, in which the MBF is able to bind to one or more components selected from the group consisting of cell attachment factors, basement membrane moieties, extracellular matrix components, and soluble circulating proteins.

3. An MBF according to Claim 1 or Claim 2, in which the modification is insertion of a heparin-binding amino acid motif.

4. An MBF according to any one of Claims 1 to 3, in which the heparin-binding motif is one which is present in a fibroblast growth factor, heparin-binding epidermal growth factor, vitronectin, fibronectin, histidine-rich glycoprotein, insulin-like growth factor binding proteins (IGFBP)or purpurin.

5. An MBF according to Claim 4, in which the heparin-binding motif is derived from a heparin-binding motif of fibroblast growth factor-1.

6. An MBF according to Claim 5, in which the heparin-binding amino acid motif is derived from the region of bovine fibroblast growth factor-1 from lysine127 to glycine141.

7. An MBF according to claim 3, in which the heparin-binding amino acid motif is a consensus heparin-binding sequence based on heparin-binding epidermal growth factor and IGF-binding protein.

8. An MBF according to any one of Claims 3 to 7, in which the heparin-binding amino acid motif is present in a non-contiguous form.

9. An MBF according to any one of Claims 3 to 7, in which the heparin-binding amino acid motif is modified to maximise its polarity.

10. An MBF according to any one of Claims 3 to 9, which additionally comprises an amino acid spacer sequence.

11. An MBF according to Claim 10, in which the spacer sequence is glycine-glycine.

12. An MBF according to any one of Claims 1 to 11, in which the polypeptide bioactive factor into which changes are introduced to create an MBF does not include a sequence motif already conferring an ability to bind to negatively-charged surfaces.

13. An MBF according to any one of Claims 1 to 12, in which the polypeptide bioactive factor into which changes are introduced to create an MBF is a growth factor which stimulates proliferation, differentiation, migration or cellular activity.

14. An MBF according to Claim 13, in which the growth factor is selected from the group consisting of members of the insulin-like growth factor (IGF), transforming growth factor-β, platelet-derived growth factor, vascular endothelial growth factor and epidermal growth factor families of growth factors.

15. An MBF according to Claim 14, in which the polypeptide bioactive factor is an IGF.

16. An MBF according to Claim 15, in which the IGF is IGF-1.

17. An MBF according to Claim 16, selected from the group consisting of MBF-1, MBF-2, MBF-3 and MBF-4, as herein defined.

18. An MBF according to claim 16, selected from the group consisting of L-MBF-1, L-MBF-2, L-MBF-3 and L-MBF-4, as herein defined.

19. An MBF according to any one of Claims 1 to 18, in which the negatively-charged surface is selected from the group consisting of extracellular matrix, dextran sulphate, chondroitin sulphate, dermatan sulphate, heparan sulphates,

heparin, collagen, fibronectin, vitronectin, laminin, hydroxyapatite, anionic plastics, silicates and physiologically-compatible metals, metal alloys, ceramics, polymers and plastic-coated metals.

20. An isolated nucleic acid molecule whose sequence encodes an MBF according to any one of Claims 1 to 19.

21. An isolated nucleic acid molecule according to Claim 20, which is a cDNA.

22. An isolated nucleic acid molecule according to Claim 21, which is a sense cDNA.

23. An expression vector comprising a nucleic acid sequence according to any one of Claims 20 to 22.

24. An expression vector according to Claim 23, further comprising a nucleic acid sequence encoding a portion of porcine growth hormone (pGH) linked to the 5' nucleotide of the sequence encoding the MBF.

25. An expression vector according to Claim 24, in which the nucleic acid sequence encoding a portion of pGH includes a cleavable sequence.

26. A composition comprising an MBF according to any one of Claims 1 to 19, together with a pharmaceutically or veterinarily acceptable carrier.

27. A composition according to Claim 26, in which the carrier is suitable for topical application.

28. A composition according to Claim 26, in which the carrier is suitable for parenteral administration.

29. A composition comprising an MBF according to any one of Claims 1 to 19, together with a cosmetically acceptable carrier.

30. A composition according to Claim 29, in which the carrier is a cream, lotion, medicated body wash, powder, toothpaste, or mouthwash.

31. A composition for the enhancement of tissue remodelling or tissue repair associated with tissue trauma or wound healing, comprising an effective amount of an MBF according to any one of Claims 1 to 19, formulated with a carrier suitable for topical application.

32. A composition for alleviation of skin damage associated with ageing or with exposure to ultraviolet or ionizing radiation, comprising an effective amount of an MBF according to any one of Claims 1 to 19, together with a cosmetically-acceptable carrier.

33. A composition for the prevention or treatment of a condition associated with impaired gut function, comprising an effective amount of an MBF according to any one of Claims 1 to 19, formulated with a carrier suitable to produce an orally stable, bioactive enteral formulation.

34. A composition for the targeting and localisation of an MBF to cells or tissues, thereby to promote cell adhesion, growth, migration or activity *in vivo*, comprising an effective amount of MBF according to any one of Claims 1 to 19, formulated in a sterile injectible carrier.

35. A cell or tissue culture supplement comprising an MBF according to any one of Claims 1 to 19, together with a physiologically compatible carrier.

36. A tissue culture vessel or insert, comprising a negatively-charged surface pre-treated with an amount of an MBF according to any one of Claims 1 to 19, effective to promote adhesion, growth, migration or activity of vertebrate cells.

37. A surgical implant or prosthesis, comprising a negatively charged surface pretreated with an amount of an MBF according to any one of claims 1 to 19 effective to promote adhesion, growth, migration or activity of vertebrate cells.

38. A method of producing a recombinant MBF as defined in any one of Claims 1 to 19, comprising the steps of (a) subcloning the nucleic acid sequence encoding the polypeptide bioactive factor into a cloning vector; and

(b) subjecting the cloning vector to mutagenesis to generate a nucleotide sequence encoding an MBF,

thereby to increase the affinity of the encoded polypeptide bioactive factor for a negatively-charged surface.

39. A method according to Claim 38, in which the mutagenesis is achieved using site-directed mutagenesis.

40. A method according to Claim 38 or Claim 39, in which the mutagenesis is achieved using one or more oligonucleotide primers which are based on binding sites for negatively-charged surfaces found in other polypeptide bioactive factors, or on sequences substantially homologous thereto.

41. A method according to Claim 40, in which the polypeptide bioactive factor is a growth factor selected from the group consisting of vitronectin, 10 kilodalton gamma-interferon inducible protein, histidine-rich glycoprotein, purpurin, beta-thromboglobulin, antithrombin III, heparin cofactor II, FGF-1, FGF-2, heparin-binding epidermal growth factor, lipocortin, protein C inhibitor, fibronectin, thrombospondin, lipoprotein lipase, hepatic triglyceride lipase, vascular endothelial cell growth factor, thrombin, neural cell adhesion molecule, and glial-derived nexin.

42. A method according to any one of Claims 38 to 41, further comprising the steps of

(a) subcloning the nucleic acid sequence encoding the MBF into an expression vector;

(b) transforming the expression vector into a bacterial, yeast or tissue culture host cell;

(c) cultivating the host cell under conditions suitable to express the MBF; and

(d) isolating the MBF.

43. A method according to any one of Claims 38 to 42, in which the MBF is expressed as a fusion protein.

44. A method according to Claim 43, in which the fusion protein is expressed within inclusion bodies.

45. A method according to Claim 43 or Claim 44, in which a fragment of porcine growth hormone is linked to the

N-terminal sequence of an MBF, optionally via a cleavable sequence.

46. A method according to any one of Claims 38 to 45, comprising the step of transforming a susceptible bacterial, yeast or tissue culture cell host with a recombinant DNA plasmid which includes one or more DNA sequences capable of facilitating the expression of an MBF fusion protein.

47. A method according to any one of Claims 40 to 46, in which the oligonucleotide primer is selected from the group consisting of

Oligonucleotide primers IGFS-2' and IGFS-2'' encoding MBF-2:

Oligonucleotide IGFS-2' (54 mer)
TGCGCTCCGCTGAAAAAAAACGGTCGTTCTAAACTGGGCCCGGCTAAATCTGCT
(SEQ ID NO. 6]

Oligonucleotide primer IGFS-2'' (48 mer)
TCTAAACTGGGTCCGCGTACCCACTTCGGCCAGGCTAAATCTGCTTGA
(SEQ ID NO. 7)

Oligonucleotide primers IGFS-1' and IGFS-1'' encoding MBF-1:

Oligonucleotide primer IGFS-1' (54 mer)
5' ATGTACTGCGCTCCGAAAAAAAACGGTCGTTCTAAACTGCTGAAACCGGCTAAA 3'
(SEQ ID NO. 8)

Oligonucleotide primer IGFS-1'' (54 mer)
5' GGTCGTTCTAAACTGGGCCCGCGTACCCACTTCGGTCAGTGATGATGC**AA**GCTT 3'
(SEQ ID NO. 9)

Oligonucleotide primers IGFS-3' and IGFS-3'' encoding MBF-3:

Oligonucleotide primer IGFS-3' (57 mer)
5' ATGTACTGCGCTCCGGGTAAAAAAAACGGCCGTTCTCAGAAACTGAAACCGGCTAAA 3'
(SEQ ID NO. 10)

Oligonucleotide primer IGFS-3'' (54 mer)
5' GGTCGTTCTCAGAAAGGCCCGCGTACCCACTTCGGTCAGTGATGATGCAAGCTT 3'
(SEQ ID NO. 11)

Oligonucleotide primers IGFS-4' and IGFS-4'' encoding MBF-4:

Oligonucleotide primer IGFS-4' (48 mer)
5' TTCCGTTCTTGCGACAAACGTCAGCTGGAAAAATACTGCGCTCCGCTG 3'
(SEQ ID NO. 12)

Oligonucleotide primer IGFS-4'' (45 mer)
5' AAATACTGCGCTCCGGGTAAACGTGGCCGTTCTGCTTGATGATGC 3'
(SEQ ID NO. 13)

48. A method for promoting adhesion, growth, migration or activity of cells on a negatively-charged surface, comprising the step of growing cells in a culture medium in the presence of a negatively-charged surface pretreated with an MBF according to any one of Claims 1 to 19.

49. A method according to Claim 48, in which the cells are of vertebrate or of insect origin.

50. A method of cell culture, comprising the step of growing cells in a culture medium comprising an MBF according to any one of Claims 1 to 19.

51. A method according to Claim 50, in which the cells are of vertebrate or of insect origin.

52. A method for the enhancement of tissue remodelling or tissue repair associated with tissue trauma or wound healing, comprising the step of administering an effective amount of an MBF according to any one of Claims 1 to 19, to a subject in need of such treatment.

53. A method for the prevention or treatment of a condition associated with impaired gut function, comprising the step of administering an effective amount of an MBF

according to any one of Claims 1 to 19, to a subject in need of such treatment.

54. A method for the targeting and localisation of an MBF to cells or tissues, thereby to promote cell adhesion, growth, migration or activity *in vivo*, comprising the step of systemic or local administration of an MBF according to any one of Claims 1 to 19, to a subject in need of such treatment.

55. A method for the prevention or treatment of periodontal disease, comprising the step of administering an effective amount of an MBF according to any one of Claims 1 to 19, to a subject in need of such treatment.

56. A method of cosmetic delivery, comprising the step of administering an effective amount of an MBF according to any one of Claims 1 to 19, to the skin or hair of a subject in need of such treatment.

57. A method according to any one of Claims 52 to 56, in which the subject to be treated is a human, or is a domestic, companion or zoo animal.

58. Use of an MBF according to any one of Claims 1 to 19, in medicine or in veterinary treatment.

59. Use of an MBF according to any one of Claims 1 to 19, in the manufacture of a medicament for the treatment of a condition associated with impaired gut function or periodontal disease.

60. Use of an MBF according to any one of Claims 1 to 19, in the manufacture of a medicament for enhancement of tissue remodelling or tissue repair associated with tissue trauma or wound healing.

61. Use of an MBF according to any one of Claims 1 to 19, in the manufacture of a medicament for the targeting and localisation of an MBF to cells or tissues, thereby to promote cell adhesion, growth, migration or activity *in vivo*.

62. Use of an MBF according to any one of claims 1 to 19 in the manufacture of a tissue culture vessel or insert

thereby to promote adhesion, growth, migration or activity of vertebrate cells.

63. Use of an MBF according to any one of claims 1 to 19 in the manufacture of a surgical implant or prostheses thereby to promote adhesion, growth, migration or activity of vertebrate cells.

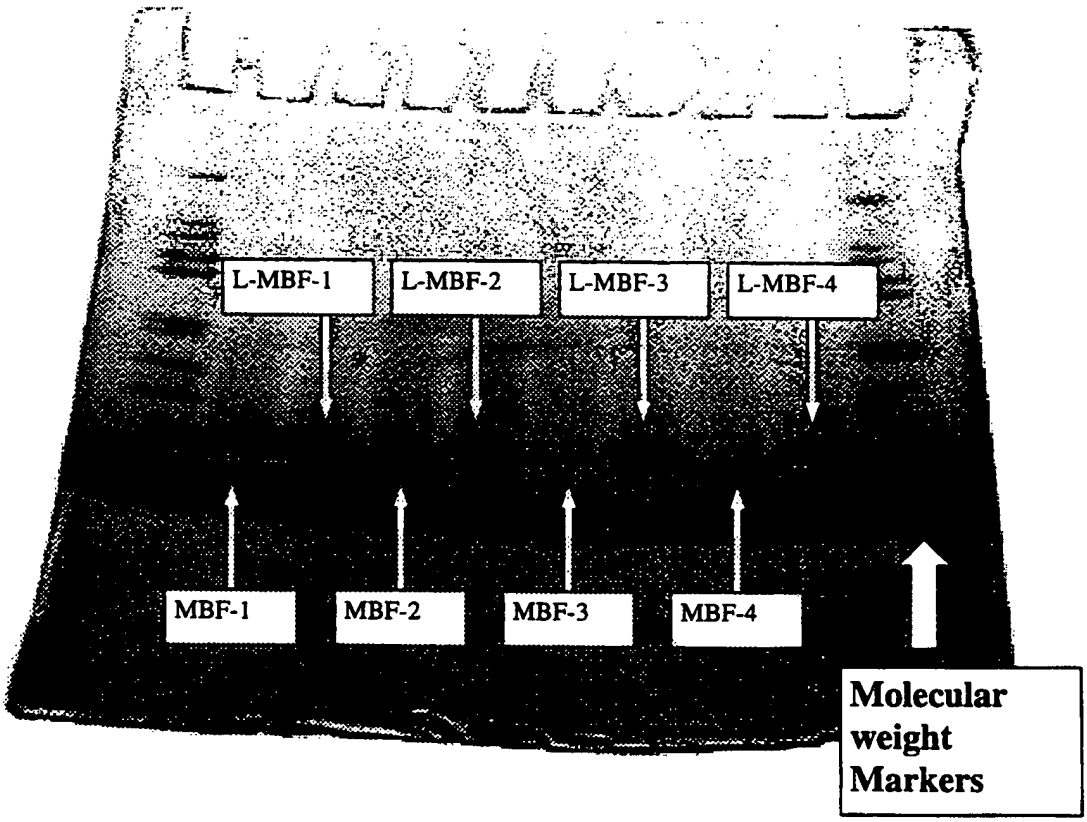
L-MBF-1
L-MBF-2
L-MBF-3
L-MBF-4
MBF-1
MBF-2
MBF-3
MBF-4
Molecular weight Markers

FIGURE 1

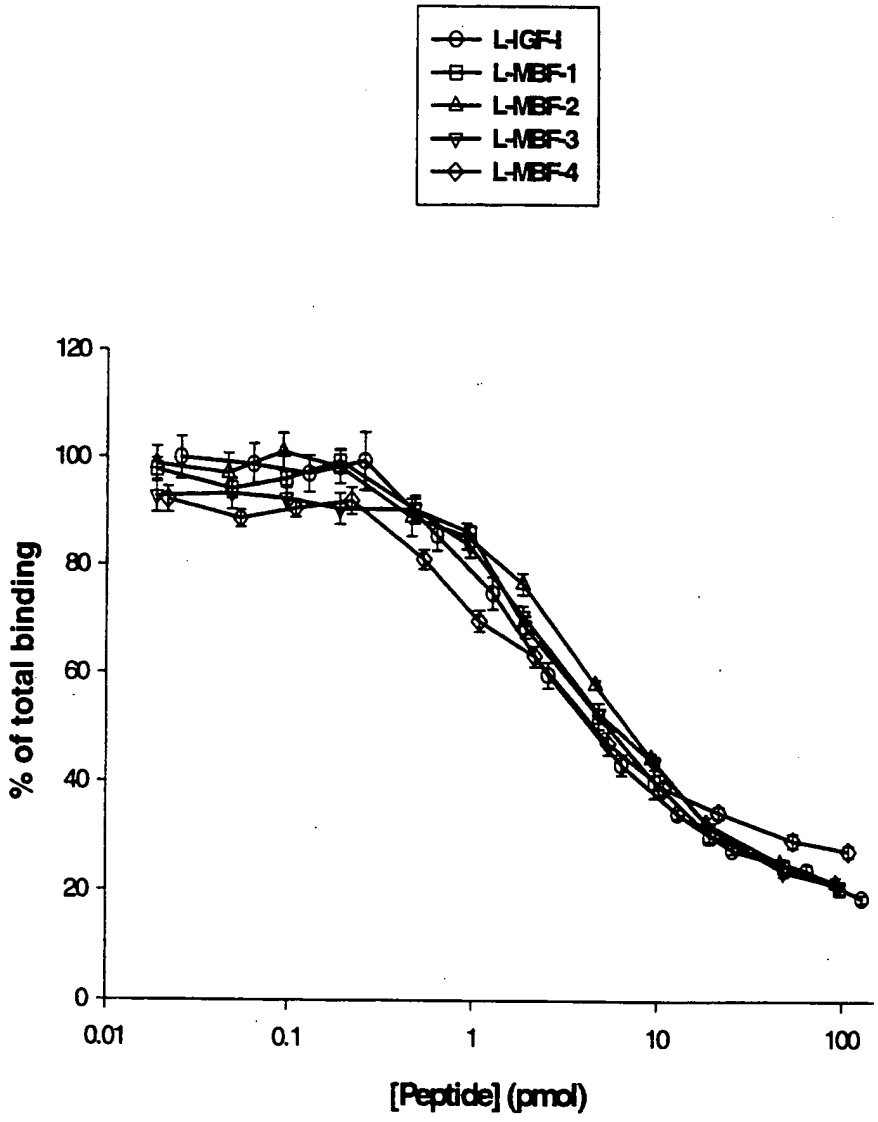
L-IGF-I
L-MBF-1
L-MBF-2
L-MBF-3
L-MBF-4
120
100
80
60
40
20
0
% of total binding
0.01
0.1
1
10
100
[Peptide] (pmol)

FIGURE 2a

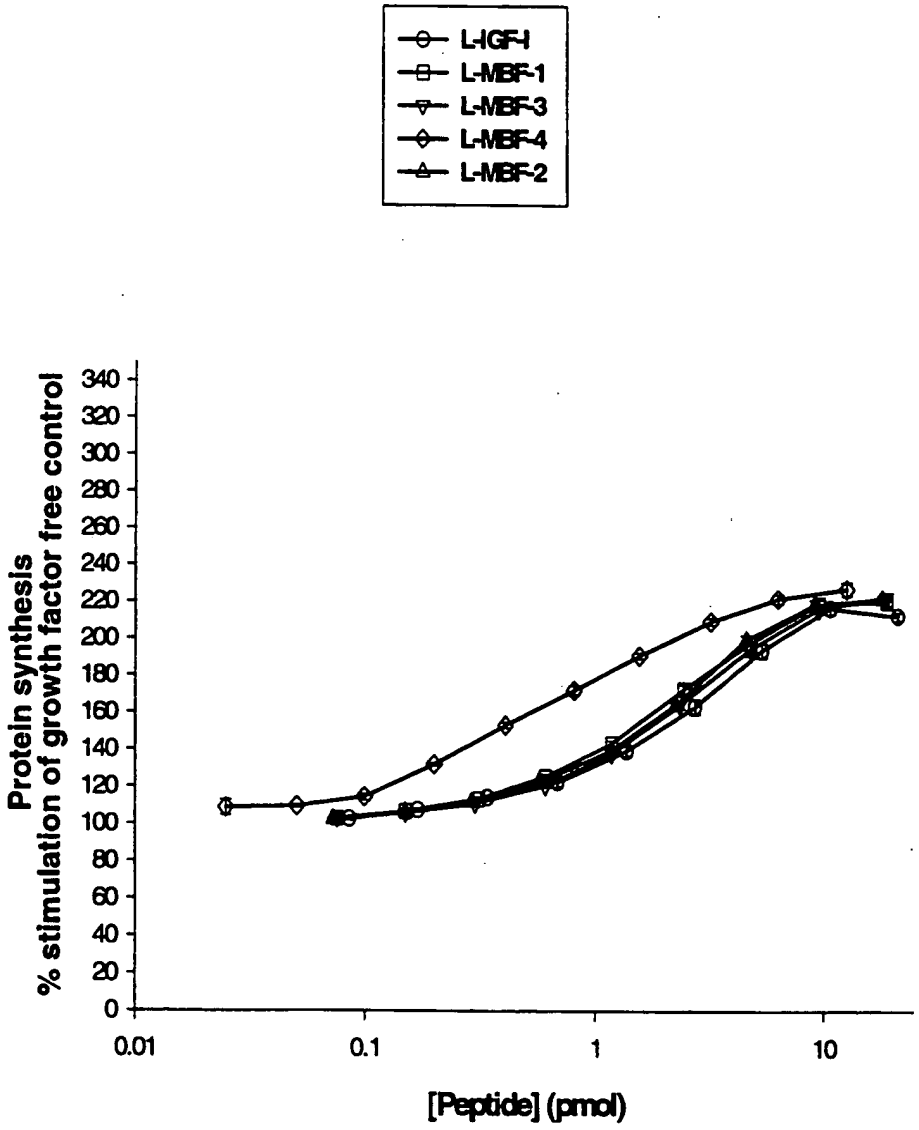
L-IGF-I
L-MBF-1
L-MBF-3
L-MBF-4
L-MBF-2
Protein synthesis
% stimulation of growth factor free control
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0.01
0.1
1
10
[Peptide] (pmol)

FIGURE 2b

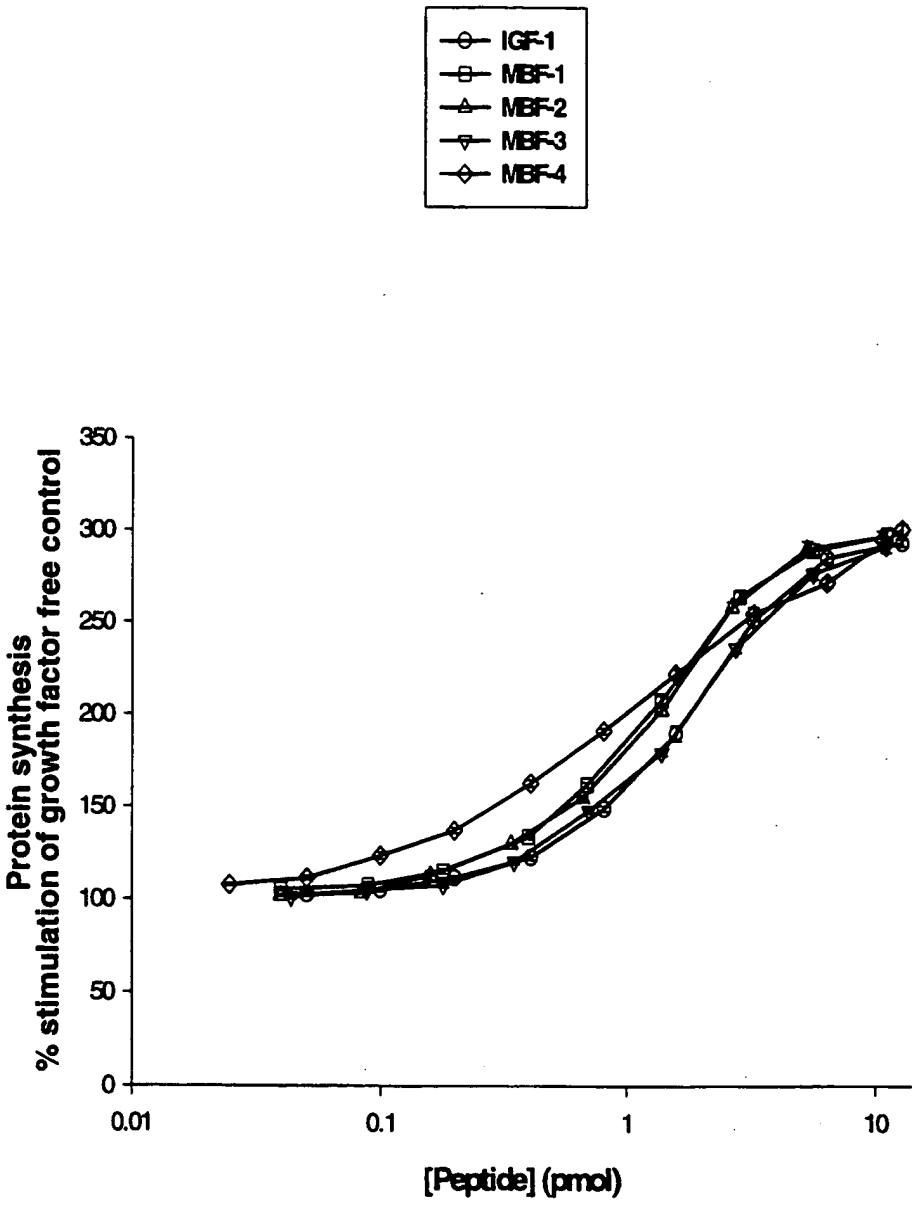
IGF-1
MBF-1
MBF-2
MBF-3
MBF-4
Protein synthesis
% stimulation of growth factor free control
350
300
250
200
150
100
50
0
0.01
0.1
1
10
[Peptide] (pmol)

FIGURE 3a

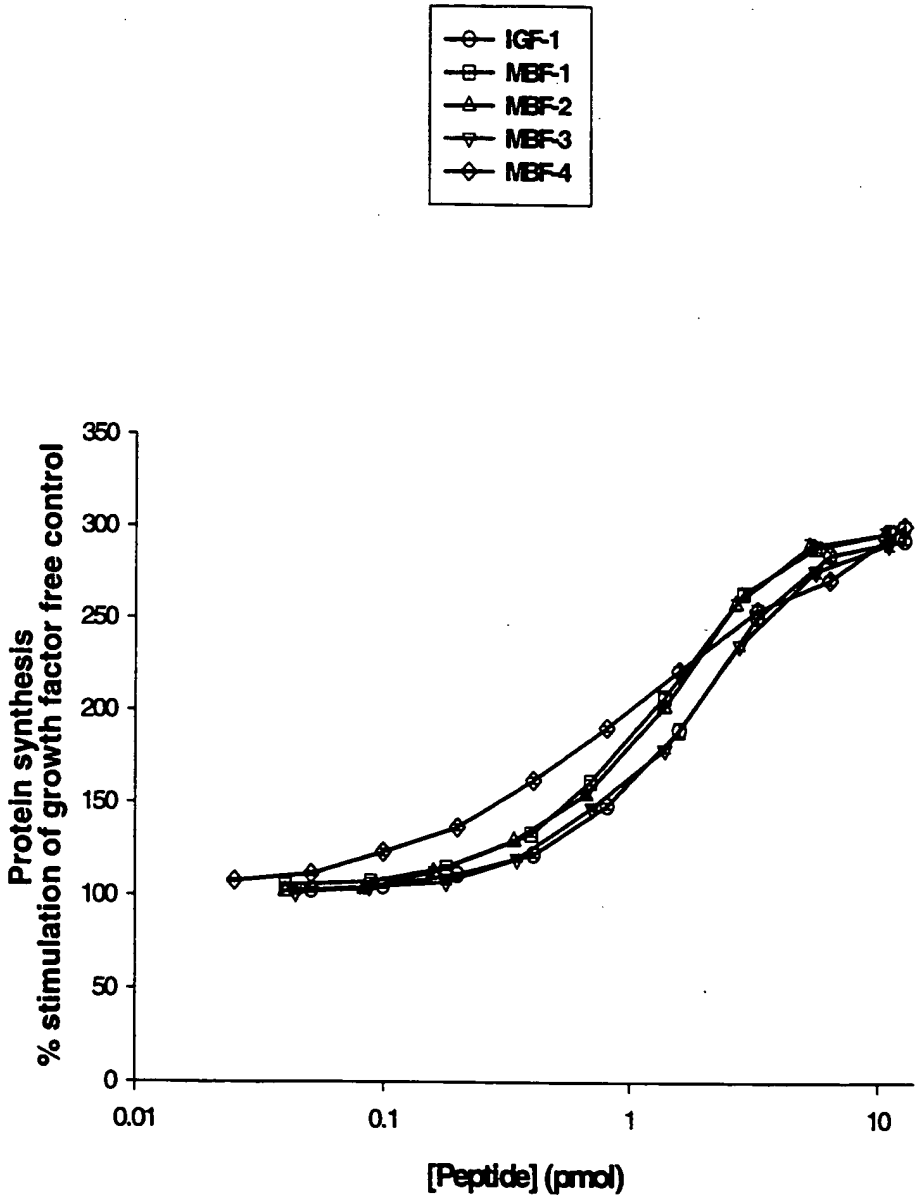
IGF-1
MBF-1
MBF-2
MBF-3
MBF-4
Protein synthesis
% stimulation of growth factor free control
350
300
250
200
150
100
50
0
0.01
0.1
1
10
[Peptide] (pmol)

FIGURE 3b

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: GROPEP PTY LTD
(B) STREET: GATE 11, VICTORIA DRIVE
(C) CITY: ADELAIDE
(D) STATE: SOUTH AUSTRALIA
(E) COUNTRY: AUSTRALIA
(F) POSTAL CODE (ZIP): 5000

(ii) TITLE OF INVENTION: MATRIX BINDING FACTOR

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: AU PP 2984

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1 5 10 15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
20 25 30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
35 40 45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Lys
50 55 60

Lys Asn Gly Arg Ser Lys Leu Gly Pro Arg Thr His Phe Gly Gln
65 70 75

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1 5 10 15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
20 25 30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
35 40 45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu
50 55 60

Lys Lys Asn Gly Arg Ser Lys Leu Gly Pro Arg Thr His Phe Gly Gln
65 70 75 80

Ala Lys Ser Ala

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1 5 10 15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
20 25 30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
35 40 45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Gly
50 55 60

Lys Lys Asn Gly Arg Ser Gln Lys Gly Pro Arg Thr His Phe Gly Gln
65 70 75 80

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1 5 10 15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
20 25 30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
35 40 45

Phe Arg Ser Cys Asp Lys Arg Gln Leu Glu Lys Tyr Cys Ala Pro Gly
50 55 60

Lys Arg Gly Arg Ser Ala
65 70

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGCGCTCCGC TGAAAAAAAA CGGTCGTTCT AAACTGGGCC CGGCTAAATC TGCT
54

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTAAACTGG GTCCGCGTAC CCACTTCGGC CAGGCTAAAT CTGCTTGA
48

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATGTACTGCG CTCCGAAAAA AAACGGTCGT TCTAAACTGC TGAAACCGGC TAAA
54

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGTCGTTCTA AACTGGGCCC GCGTACCCAC TTCGGTCAGT GATGATGCAA GCTT
54

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATGTACTGCG CTCCGGGTAA AAAAAACGGC CGTTCTCAGA AACTGAAACC GGCTAAA 57

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTCGTTCTC AGAAAGGCCC GCGTACCCAC TTCGGTCAGT GATGATGCAA GCTT 54

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TTCCGTTCTT GCGACAAACG TCAGCTGGAA AAATACTGCG CTCCGCTG
48

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAATACTGCG CTCCGGGTAA ACGTGGCCGT TCTGCTTGAT GATGC
45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00292

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 19/00, 14/65; C07H 21/04; C12N 15/62, 15/12, 15/19, 15/16, 9/20, 9/74; A61K 38/18; A61L 27/00

According to International Patent Classification (IPC) or t both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN : File CA, MEDLINE, WPIDS, Keywords (Insulin-like growth factor or IGF?) AND HEPARIN AND (MODIF? OR MUTA? OR DELET? OR ALTER? OR SUBSTIT?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochimica et Biophysica Acta, 1243, (1995), 538-542, L.F. Congote, "Increased heparin binding by site directed mutagenesis of a recombinant chimera of bombyxin and insulin-like growth factor II" see whole article	1-63
X	American Journal of Physiology, 273, (1997), E1005-13, P.G. Campbell et al., "Insulin-like Growth Factor Binding Protein-5- (201-218) region regulates hydroxyapatite and IGF-I binding" see whole document	1, 2, 12, 13

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 1999	10 JUN 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer L.F. M°CAFFERY Telephone No.: (02) 6283 2573

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00292

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Growth Regulation, (1996), 6, 206-213, B.A. Booth et al., "Structure Function Relationships in the Heparin-binding C-terminal region of insulin-like Growth Factor Binding Protein-3" see whole document	1, 2, 12, 13
X	Biochemical and Biophysical Research Communications, 240, 707-714, (1997), J.A. Koedam et al., "Insulin-like Growth Factor Binding Proteins-3 and -5 Form Sodium Dodecyl Sulfate-Stable Multimers" see whole document	1, 2, 12, 13
X	J. Biol. Chem., 271(11), 6099-6106, 1996, T. Arai et al., "Substitution of Specific Amino Acids in Insulin-like growth Factor Binding protein-5 Alters Heparin binding and its Change in Affinity for IGF-I in response to Heparin" see whole document	1, 2, 12, 13
X	Endocrinology, 138 (7), 2972-2978, 1997, T.J. Nam et al., "Insulin-like growth factor binding protein-5 binds to plasminogen Activator inhibitor-1" see whole document	1, 2, 12, 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00292

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1-14, 20-47, 48-63**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The scope of the claims is such that a complete search cannot be carried out economically. Accordingly the present search is limited to modification of insulin-like growth factor by heparin-binding moieties.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.